Osteoprotegerin in Fibrotic Disorders

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Chapter I

GENERAL INTRODUCTION

AIM AND SCOPE OF THE THESIS

Clinical studies have reported higher serum level of OPG in cases of fibrosis, especially liver fibrosis[1-3], but there is limited data available to explain the significance of this protein in the biology of the disease. This thesis is aimed to give wider insight into this topic.

In **chapter 2**, we studied the possible roles OPG could have in liver fibrosis using human samples, mouse models of liver fibrosis, murine precision-cut liver slices, and relevant cell lines. These studies included upregulation of its production by TGFβ and investigation into its role as a decoy receptor of RANKL. In addition, we found that OPG itself can upregulate the expression of TGFβ, which suggests that OPG and TGFβ are involved in a feed-forward loop. In **chapter 3** we studied whether the role of OPG in liver fibrosis could be extended to a more general role in fibrotic processes. We investigated whether induction of fibrosis in other organs (lung, kidney, colon) also resulted in increased OPG production using precision-cut slices of these organs. Furthermore, we studied how OPG responds to antifibrotic treatment in the different organ slices. In **chapter 4** we further studied regulation of OPG by different cytokines and found that in addition to TGFβ, stimulation with IL13 could also induce expression of OPG. We further investigated how IL13 can induce OPG production and found this is also mediated through TGFβ. Hypothesizing a feedback mechanism controlling the TGFb-OPG feed-forward loop, in **chapter 5**, we investigated the influence of miR-145-5p in controlling the OPG-TGFβ profibrotic loop.

OPG has an important role in the regulation of bone formation and degradation by inhibiting osteoclast activation and function in bone extracellular matrix degradation. We hypothesized a similar role for OPG in fibrosis by inhibiting antifibrotic macrophages and we therefore in **chapter 6** investigated literature to see what is known about antifibrotic macrophages and how we could look at those in the future studies involving OPG.

Finally, we discuss and summarize our findings in **chapter 7** with future perspectives of the potential use of OPG as a new therapeutic target or biomarker for (liver) fibrosis.

FIBROSIS

Fibrosis is abnormal fibrogenesis characterized by the accumulation of extracellular matrix molecules such as collagens and fibronectin produced by myofibroblasts that are characterized by increased expression of **α**-smooth muscle actin (**α**SMA)[4,5]. The pathology of fibrosis is marked by the unbalanced regulation of inflammation, matrix formation, and resolution, resulting in maintaining persistent formation of non-functional matrix with no or very slow resolution towards functional tissue replacement[6,7]. The disbalance can be caused by several factors, which can be related to each other: (1) continuous signalling from the injury or infection that trigger immune responses[8], (2) irregular cellular functions or differentiation, for examples of macrophages and fibroblasts[9,10], and (3) homeostatic abnormalities caused by several possible factors such as a genetic disorder[11,12] or the absence of blood vessels at the site of fibrosis[13].

In most cases, fibrosis is associated with organ failure as its advanced pathological event[14], and organ transplantation is then the only option for treatment[15]. Much effort has been put in by researchers in understanding the biology and cause of organ fibrosis in order to find a strategy to prevent and to cure. However, to date no effective treatment has been found yet[16]. In some specific types of fibrosis such as cystic fibrosis and familial pulmonary fibrosis, heredity is the cause of the disease[17,18]. However, in most other cases of fibrosis, the disease develops over a lifetime and the exact cause cannot be determined anymore[19,20]. Although there are some risk factors like chronic infection and chemical exposures that are known to increase the occurrence rate, it has not been proven yet whether they are the main cause of the disease.

Fibrosis can occur in many organs, including vital organs like the liver, the lungs, the heart, the intestines and the bone marrow. Of those organs, pulmonary and liver fibrosis are most prevalent[21,22,23] and researchers and clinicians are trying to find a way to detect the disease in the earliest phase possible in order to prevent or to avoid an incurable stage of the disease.

LIVER FIBROSIS

Liver fibrosis is a burden to mankind, contributing to almost 2% of global deaths in 2010[24]. The pathology of the disease is characterized by the accumulation of extracellular matrix consisting of collagens and other matrix proteins in the liver, preventing regeneration of new functional parenchymal cells[25]. Liver fibrosis is thought to be the result of unbalanced wound healing process caused by several chronic factors such as hepatitis infection, alcohol abuse, or long-term use of hepatotoxic drugs (figure 1)[26].

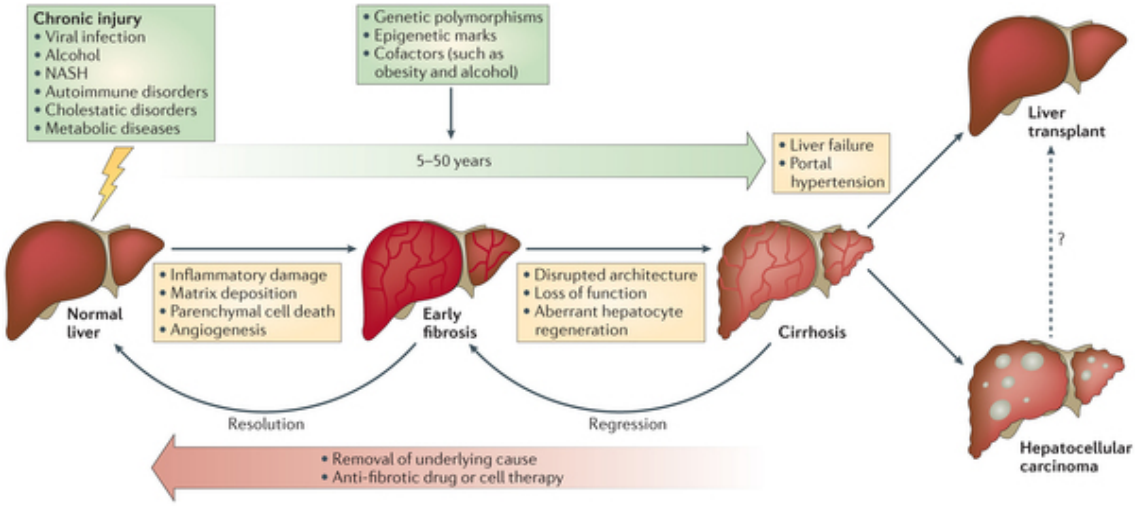

Figure 1. *Schematic of liver fibrosis progression and resolution as summarized by Pellicoro et al. (2014). The disease is hypothesized to start with chronic inflammation followed by loss of function and can ultimately end in cirrhosis[26].*

Fibrotic sites in the liver are dominated by activated hepatic stellate cells instead of hepatocytes and these stellate cells produce profibrotic cytokines and growth factors to maintain the state of fibrosis[25]. The early state of liver fibrosis is hard to diagnose since symptoms of disturbed liver function only occur when the liver is moderately to severely compromised[27]. Although it is considered possible to reverse the fibrotic process towards resolution[28], to date there is no effective medical treatment available that can do this, especially in cases of full-blown cirrhosis[29]. Therefore, liver transplantation is currently the only option to restore organ functionality in time.

The pathogenesis of liver fibrosis includes, but is not limited to, three main factors: (1) persistent inflammatory and immune responses by macrophages and lymphocytes from long-term injury[30], (2) hepatocyte death either by apoptosis or necrosis and replacement by fibroblasts[31], and (3) high oxidative stress that triggers stellate cells activation, this is especially the case for fibrosis caused by infections and chemical injury[32]. The process of liver fibrosis is generally thought to start with continuous inflammation that activates Kupffer cells, liver resident macrophages, to release various proinflammatory and profibrotic cytokines such as TNFα, IL1β, TGFβ, and PDGFBB[33]. This is followed by activation of hepatic stellate cells and transformation of these cells into myofibroblasts that are the main producers of the excess extracellular matrix that characterizes fibrosis[34]. The activation of hepatic stellate cells is prominent and therefore is the most studied event for drug development purposes[35,36].

LIVER FIBROSIS RESOLUTION

It is a matter of debate among scientists whether the process of liver fibrosis can be reversed, especially when the disease has already developed. Current liver fibrosis medication is aimed at inhibiting inflammation to stop oxidative stress and other inflammatory stimulants and is thereby expected to consequently protect the remaining functional hepatocytes. Resolution is then expected to occur spontaneously. As inflammation is thought to be important in the process of fibrosis, the use of anti-inflammatory drugs, mainly corticosteroids, is widely proposed [37,38]. However, this approach appears not to be effective and casts doubts on the importance of inflammation in the later stages of fibrosis[39]. Recently, there are some other approaches under development, including degrading or altering the composition of extracellular matrix[40], inhibiting further hepatic stellate cells (HSCs) activation[41], supporting hepatocytes growth using hepatocyte growth factor (HGF)[42], and directing macrophages differentiation towards antifibrotic phenotypes[43]. With these approaches, some experimental studies showed promising results towards resolution of liver fibrosis, marked by the lower expression of some frontline markers like collagen and αSMA[44].

As liver fibrosis is often represented as the activity of HSCs, the progression as well as resolution of liver fibrosis can be followed by simply evaluating the activation and deactivation or apoptosis of HSCs[41]. Both directions involve communication with lymphocytes and macrophages, mediated by several prominent mediators such as IFNγ, TGFβ, IL13, and MMP9. Aforementioned markers such as collagen-1 and αSMA can be used to evaluate changes in HSCs activation and deactivation/apoptosis *(figure 2)*[26].

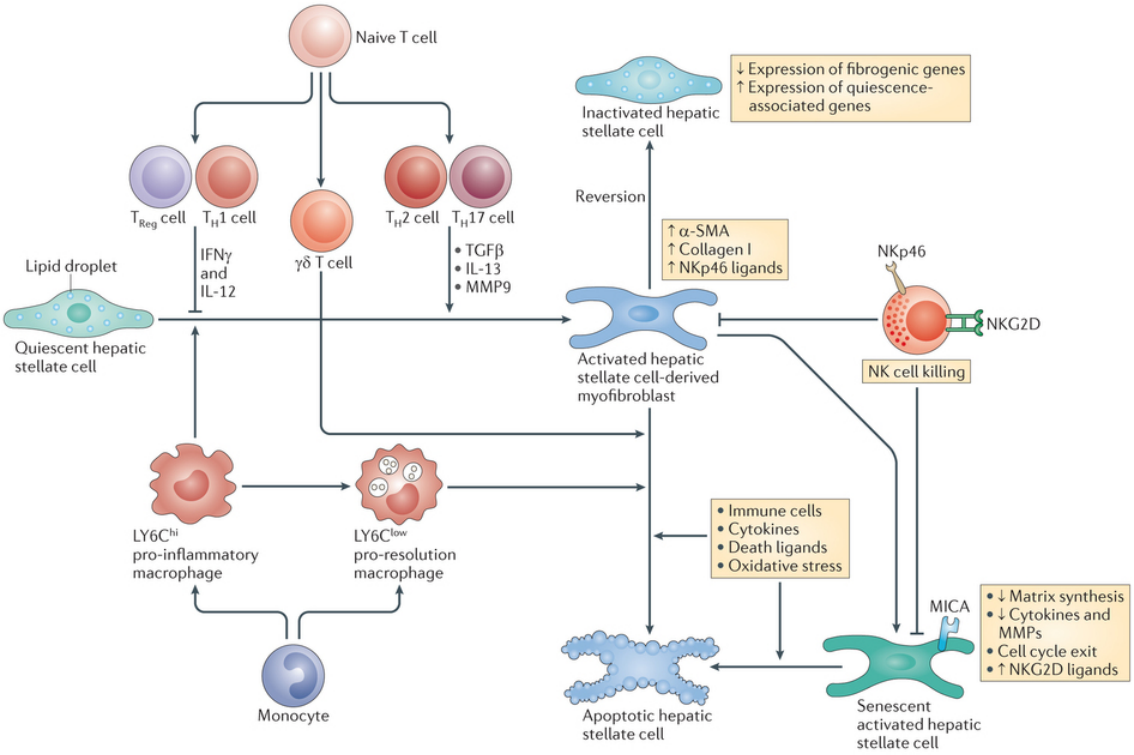

Figure 2. *Liver fibrosis progression and resolution can be represented by HSCs activation and reversion. The process involves T cells and macrophages, communicating via various cytokines and growth factors with HSCs, and the process can be evaluated using several markers expressed by HSCs. The figure is republished with permission*[26].

The proposed approaches to stimulate liver fibrosis resolution are generally to support antifibrotic machineries and to suppress profibrotic ones. In order to interfere within a persistent fibrotic condition and to induce resolution, knowledge of how the involved cells communicate with each other during normal resolution is essential. Targeting the key mediators involved in this cellular communication during fibrosis may solve the question how resolution can be brought about by medical effort. In order to reach that goal, better

understanding of the roles and activities of the various communication mechanisms is important.

Since liver fibrosis is a chronic process involving immune responses, inflammation, and wound healing, many soluble mediators have been reported to take part in the biology of the disease. The mediators can be divided into functional subgroups: Chemokines such as CCL2, CCL3, CCL17, CCl22, CXCL9, and CXCL10 that are responsible for cell recruitment to the site of fibrosis[45-47]; immune and inflammatory cytokines such as IL1β, TNFα, IFNγ, and IL33 that are responsible for oxidative stress as well as macrophage and lymphocyte activation[48]; and growth factors, cytokines and enzymes involved in tissue remodelling such as TGFβ, PDGF, IL4, IL13, fibronectin, tissue transglutaminase, MMP9, and MMP13 that are responsible for the activation of fibroblasts, tissue regeneration, and resolution of fibrosis[49-54]. There are some proteins or compounds with the increased expression or production during liver fibrosis but not considered as mediators, because these are components of the extracellular matrix itself i.e. collagens, glycoproteins, and hyaluronic acid, which are also then considered as biomarkers[55]. TGFβ has been reported to be responsible for many profibrotic signals especially in liver fibrosis albeit it also has essential functions in homeostasis[56]. TGFβ signalling through the SMAD pathway activates hepatic stellate cells and contributes to lipid accumulation, therefore supports inflammation and maintains extracellular matrix[54]. Moreover, TGFβ also contributes to hepatocyte cell death and increasing ROS production to induce oxidative stress[57].

Recent reports have also shown that the bone matrix-related protein osteoprotegerin (OPG) in blood is increased during liver fibrosis and can serve as a biomarker to increase diagnostic accuracy of tests to diagnose liver fibrosis[1-3]. Although involved in bone matrix regulation, OPG itself is not an extracellular matrix component and its role in liver fibrosis has not been elucidated. Interestingly, a recent study has reported that TGFβ can induce OPG production in synovial fibroblasts in arthritis[58]. This information suggests a link between OPG and fibroblasts and thereby suggests a potential role in the pathogenesis of liver fibrosis.

OSTEOPROTEGERIN IN FIBROSIS

Osteoprotegerin (OPG), also known as tumor necrosis factor receptor (TNF) superfamily 11B (TNFRS11B), has been widely studied as a decoy receptor of RANKL, also known as TNF ligand superfamily 11 (TNFSF11), and TRAIL, also known as TNF ligand superfamily 10 (TNFSF10)[59]. OPG was first discovered to be produced by osteoblasts and regulates bone formation and remodelling by binding to RANKL thus preventing osteoclast activation[60]. Osteoprotegerin is produced as a 60 kDa-monomer consisting 401 amino acids. The monomers may also be assembled at the cys-400 residue to form 120 kDa disulphide-linked dimers. Both monomer and dimer proteins have a signal peptide, which is cleaved prior to secretion to form active OPG. As a decoy receptor for RANKL and TRAIL, the structure of OPG consists of four cysteine-rich pseudo repeats located in the N-terminal, responsible for its binding activity to RANKL and TRAIL. However, it lacks a trans-membrane domain for attachment to cell membranes and is thus biologically available as a soluble protein, which increases its effectiveness to catch RANKL and TRAIL available in the environment[61,62].

Further studies have shown that OPG is not only produced by osteoblasts, but also smooth muscle cells, fibroblasts and cancer cells and is postulated to have a significant role in arthritis and cancer[59,60,63]. In cancer, for instance, OPG is produced by cancer cells to intercept TRAIL to avoid TRAIL-receptor mediated apoptosis[64], while in arthritis OPG was shown to be produced by synovial-like fibroblasts to maintain their activated state as well as to avoid apoptosis[65].

Recent studies have also reported OPG to be higher in several types of organ fibrosis. Garcia-Valdecasas-Campelo (2006) reported higher serum OPG levels in patients with liver fibrosis and alcoholic liver disease[1], Boorsma *et al*. (2015) in patients with pulmonary fibrosis[66], and Sen *et al.* (2005) in patients with postoperative epidural fibrosis[67]. Including OPG as an additional biomarker to a panel of biomarkers to diagnose liver fibrosis has been introduced by Bosselut *et al*. (2013) and proven to increase the diagnostic accuracy of their panel[3]. However, it is not clear what role OPG plays in fibrotic processes in general and liver fibrosis in particular.

In addition to the hypothesis of preventing TRAIL-induced apoptosis of activated myofibroblasts, another possible hypothesis is that OPG can interfere with break-down of extracellular matrix. Meng *et a.l* reported that OPG directly inhibits the production of matrix metalloproteinase-13 (MMP13)[68], an important

antifibrotic MMP in liver fibrosis[69]. Moreover, Corisdeo *et al.* (2001) suggested in their study that RANKL stimulates the production of cathepsin K, a collagen-degrading protease, in bone marrow cultures and macrophages[70]. This finding suggests that high levels of OPG can prevent expression of MMP13 and cathepsin K in other cells like macrophages and thus inhibit ECM degradation. Furthermore, Toffoli *et al.* (2011) showed that OPG could promote vascular fibrosis by inducing TGFβ1 production in vitro and in vivo[71].

However, despite the evidence of the possible profibrotic activities of OPG, there are no studies explaining how OPG contributes to the development of organ fibrosis especially in the liver. As a soluble receptor, there is very limited evidence of OPG directly interacting with a membrane receptor, therefore any profibrotic activity of OPG is mostly like explained by its abilities to scavenge ligands like RANKL and TRAIL.

MICRORNA AND THEIR ROLES IN FIBROSIS

MicroRNAs (miRNAs) are non-coding small RNA molecules that can degrade or block translation of their target messenger RNA (mRNA) sequences. This is done in collaboration with the protein Argonaut in a complex called RNA-induced silencing complex (RISC)[72]. miRNAs were first discovered by Lee *et al.* (1993) and Wightman *et al.* (1993) when studying negative regulation of lin-14 in *Caenorhabditis elegans* through formation of small molecule RNA later described as microRNA[73,74]. From then on, miRNAs have been studied widely as feedback mechanisms of many biological mechanisms in various diseases especially in cancer and fibrosis[75]. Particularly during fibrosis, miRNAs can be either up- or down-regulated, and thereby contribute to the pathogenesis, progression, and resolution of fibrosis *(figure 3)*[76,77].

The role of OPG in liver fibrosis is very likely to also involve microRNAs in some way. Ong *et al.* have recently reported microRNA expression levels in human fibroblasts with or without TGFβ treatment, and found that some of the TGFβ-upregulated miRNAs can target OPG, one of those being miR145-5p[78].

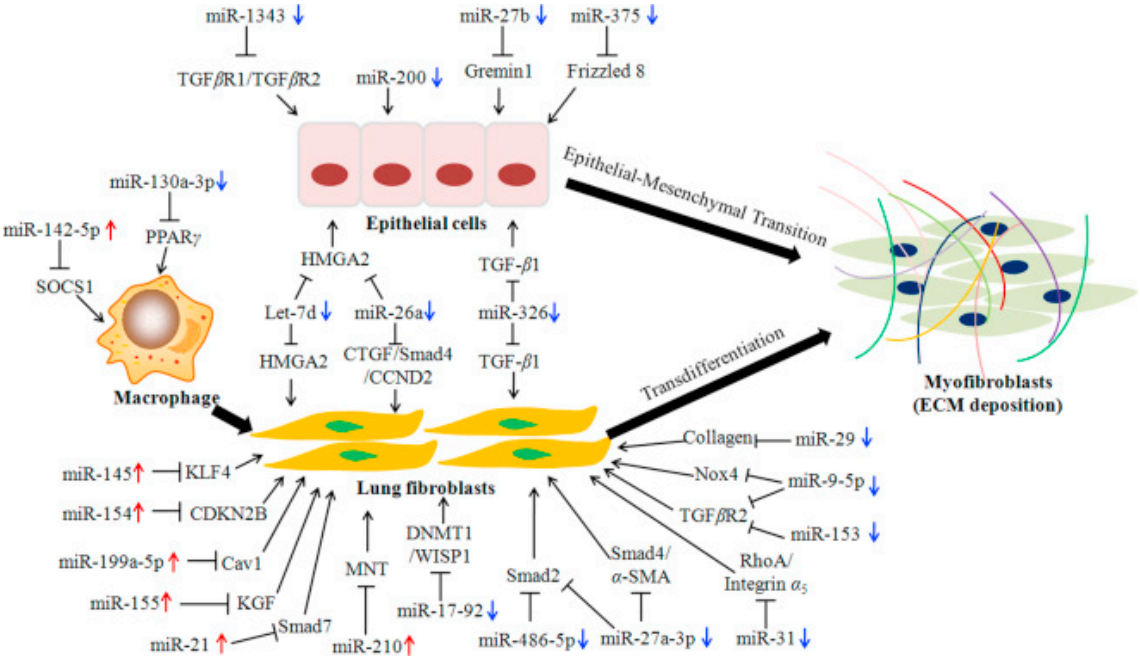

Figure 3. *The up- and down-regulation of miRNAs in idiopathic pulmonary fibrosis (IPF) as summarized by Li et al. (2016). As miRNAs can block cytokine expression, miRNAs can play pro- and antifibrotic roles in fibrosis. MiRNAs can interfere with the activities of many cells like epithelial cells, macrophages, and fibroblasts. Changes in the expression of miRNAs during fibrosis can alter homeostasis and therefore, these molecules may be potential targets for therapy. Figure is reprinted with permission*[77].

At least three different recent studies have reported effects of miR-145 on expression of OPG. Jia *et al.* (2017) reported that transfecting human osteoblast-like MG-63 cells with miR-145-5p mimics can partially inhibit OPG upregulation by estrogen[79]. In another study, Wang *et al.* (2017) confirmed OPG as a direct target for mir-145 by using a dual-luciferase reporter assay and further showed that higher mir-145 expression can suppress OPG expression[80]. Finally, Zhao *et al.* (2016) showed that OPG expression was significantly lower after lentivirus-mediated transfection of rats and THP-1 cells with miR-145[81].

As miR-145 obviously targets OPG, it may also be involved in regulation of OPG production in fibroblasts. Interestingly, Yang *et al.* (2013) reported upregulation of miR-145 in TGFβ1-treated lung fibroblasts as compared to untreated cells[82]. Furthermore, Zhou *et al.* (2016) reported in their study that miR-145 inhibited TGFβ-induced human and rat hepatic stellate cells activation and proliferation in vitro and downregulation of this microRNA in vivo in CCl_4-induced liver fibrosis in mice[83].

Based on literature reports, we hypothesized that OPG is produced by fibroblasts in liver tissue and that OPG is associated with fibrotic processes because its expression is controlled by TGFβ. In addition, this expression may be further regulated by microRNAs, especially miR-145.

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